Subcellular Targets of Cadmium Nephrotoxicity: Cadmium Binding to Renal Membrane Proteins in Animals With or Without Protective Metallothionein Synthesis

Gunnar F. Nordberg,[1] Taiyi Jin,[1] and Monica Nordberg[2]

[1]Department of Environmental Medicine, University of Umeå, Umeå, Sweden; [2]Department of Environmental Hygiene, Karolinska Institute, Stockholm, Sweden

Nephrotoxic effects of cadmium exposure are well established in humans and experimental animals. An early manifestation of such toxicity is calciuria a few hours after injection of CdMT in rats. Protection against calciuria and other adverse effects such as proteinuria (occurring later) is offered by pretreatment with Cd, which effectively induces metallothionein synthesis. In the present experiment, one group of animals was given pretreatment with $CdCl_2$ to induce metallothionein synthesis. The comparison group was left without pretreatment. The distribution of Cd from a normally nephrotoxic dose of ^{109}CdMT was studied by gel chromatography in subcellular fractions of kidney cortex in both groups. In the pretreated animals, ^{109}Cd in the plasma membrane and microsome fractions of renal cortical cells was mainly bound to metallothionein and other low molecular weight proteins at 4 hr. In nonpretreated animals the major part of ^{109}Cd was bound to high molecular weight proteins. These findings indicate that membrane proteins may be important targets for Cd when inducing nephrotoxicity and that sequestering of Cd by metallothionein (and other low molecular weight proteins) may be a mechanism of protection. — Environ Health Perspect 102(Suppl 3):191–194 (1994)

Key words: metallothionein, toxicity, gel chromatography, subcellular fraction, plasma membrane, microsomes, kidney, cadmium

Introduction

Renal tubular dysfunction may develop in experimental animals and in humans as a result of long-term exposure to Cd (1). After absorption via pulmonary or gastrointestinal routes, Cd is transported in plasma initially bound to albumin and other larger proteins (2). Cadmium-albumin is taken up predominantly by the liver and Cd is released from albumin in this organ. The released Cd induces synthesis of metallothionein in liver; and some time after uptake in the liver, most Cd is bound to metallothionein (3). A small proportion of metallothionein-bound Cd will be released into plasma, and because of its low molecular weight, it will be efficiently transported through the glomerular membrane and will reach the tubular fluid in the kidney. It will be further taken up by pinocytotic vesicles in the brush border of

the proximal tubule and transported into lysosomes where metallothionein is split from Cd (4–6). The released Cd is believed to constitute the toxic principle in cell. The rate at which such Cd is bound to newly synthesized metallothionein in renal cells is believed to be a key factor in determining whether or not renal tubular damage occurs (7–9). Pretreatment with $CdCl_2$, which effectively induces the synthesis of metallothionein in renal cells, prevented acute nephrotoxicity from normally occurring after an injected challenge dose (0.4 mg/kg) of Cd-metallothionein (10). These observations gave general support to the idea that intracellular metallothionein can prevent toxicity by sequestering Cd from sensitive targets. However, it has not previously been demonstrated which cellular components are the critical targets for Cd in the renal cells, and it has thus been difficult to demonstrate the role of metallothionein-bound and nonmetallothionein-bound Cd in relation to development of renal damage. In recent studies (11,12), it was shown that an increased urinary Ca excretion is an early manifestation of Cd nephrotoxicity in the Cd-metallothionein injection model and that such toxicity can be prevented by pretreatment with $CdCl_2$. The aim of the present study was to try to identify the subcellular targets of Cd nephrotoxicity by

studying the binding of Cd to various proteins in subcellular fractions from the renal cortex of rats. By examining possible differences in such binding between animals with or without protective pretreatment, the importance of various targets for expression of toxicity could be indicated. Previously developed models for induction of nephrotoxicity and protective pretreatment (10,12) were employed.
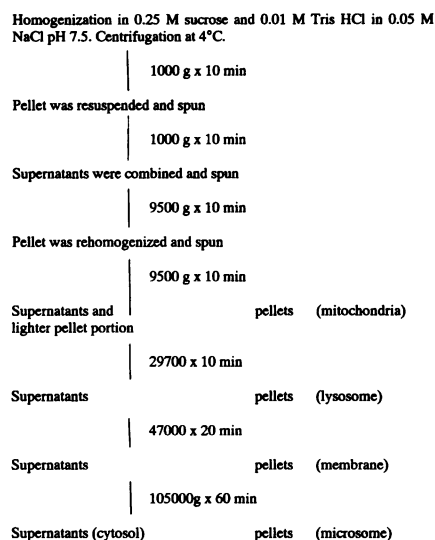
Materials and Methods

Animals

Male Wistar rats (200–250 g) from ALAB (Stockholm, Sweden) were used in this study. All animals were allowed to acclimatize for a minimum of 7 days prior to the experiment; they were maintained under controlled environmental conditions of temperature and humidity and with a 12-hr day-night cycle (2 A.M.–2 P.M.). Tap water and food (Ewos-ALAB rat-mouse food R3) were provided *ad libitum*. Four groups of rats, each consisting of two animals, were studied. Groups A and B were not pretreated with Cd but were subcutaneously injected with normal saline. The rats in groups C and D were given subcutaneous pretreatment injections with $CdCl_2$ in doses increasing in the following order: 0. 5, 1, 1, 2, 2 mg Cd/kg during 5 consec-

Homogenization in 0.25 M sucrose and 0.01 M Tris HCl in 0.05 M NaCl pH 7.5. Centrifugation at 4°C.

| 1000 g x 10 min

Pellet was resuspended and spun

| 1000 g x 10 min

Supernatants were combined and spun

| 9500 g x 10 min

Pellet was rehomogenized and spun

| 9500 g x 10 min

Supernatants and   pellets   (mitochondria)
lighter pellet portion

| 29700 x 10 min

Supernatants   pellets   (lysosome)

| 47000 x 20 min

Supernatants   pellets   (membrane)

| 105000g x 60 min

Supernatants (cytosol)   pellets   (microsome)

Figure 1. Preparation of subcellular fractions from rat kidney cortex.

utive days. A challenge dose of ^{109}CdMT (0.4 mg Cd/kg, sc) was given to each animal in all groups 48 hr after the last pretreatment injection. All injections were given at 8 A.M. Animals in groups A and B were sacrificed by cervical dislocation 1 hr after, and those in groups C and D, 4 hr after injection of ^{109}CdMT. The kidneys were immediately excised and used for the preparation of subcellular fractions.

Subcellular Fractionation

The subcellular fractions were prepared (Figure 1) by a differential centrifugation method described by Maunsbach (13). The kidney cortex was dissected into small pieces and homogenized at 800 rpm and 100 strokes with a glass-Teflon homogenizer in cold 0.25 M sucrose and 0. 01 M Tris-HCl buffer in 0. 05 M NaCl, pH 7.5. The homogenate was centrifuged at 1000g for 10 min. The pellets were rehomoge-

nized and spun at 1000g for another 10 min. The resulting pellet was designated the nuclear fraction (1000g). The supernatants were combined and spun at 9500g for 10 min. The pellets were resuspended and spun at 9500g again. The resulting pellet was used as mitochondria fraction (9500g). The supernatants and lighter pellet portion were combined and spun at 29,700g for 10 min. The pellet obtained was designated the lysosome fraction (29,700g). The supernatants were spun at 47,000g for 20 min. This pellet was the plasma membrane (47,000g). The supernatant was centrifuged at 105,000g for 60 min. Centrifugation was performed at 4°C. The pellet was used as microsome fraction (105,000g) and the supernatant as the cytosol fraction. ^{109}Cd content in the subcellular fractions was determined by Packard gamma scintillation system (Packard Instrument Co.) as described by Nordberg and Nishiyama (14).

Chemicals

Tris-(hydroxymethyl)-aminomethan was from Merck (Germany). ^{109}Cd was purchased from Amersham (UK). DOC (Deoxycholic acid sodium salts) was from Fulka Biochemicka (Switzerland). ^{109}CdMT was prepared from rat liver as described by Nordberg et al. (15). The metallothionein used in the present study had a Cd/Zn ratio of 37.0/13.5 and a E250/E280 ratio of 3.92.

Preparation of Subcellular Protein Complexes

The subcellular fractions were solubilized with 1% sodium deoxycholate (DOC) by stirring for 15 min in an ice-cold bath under a nitrogen stream. Each fraction was adjusted to contain 1.5% protein. Each solubilized fraction was applied to a column packed with Sephadex G-75 (26 × 364

mm). The column was eluted with 0.01 M Tris-HCl 0.05 M NaCl buffer, pH 7.5, containing 0.1% DOC and performed at 4°C. A portion of each fraction (3.5 ml) was assayed for absorbance at 250 nm and ^{109}Cd was estimated by γ-counting. The recovery of ^{109}Cd was 84 to 102% of the amount applied to the column.

Results

In the present studies metallothionein synthesis was induced in renal cells by a previously tested pretreatment schedule with nonradioactive Cd (10). A challenge dose of radiolabeled ^{109}CdMT, sufficient to induce renal damage with proteinuria in nonpretreated animals (10,11), but not in pretreated animals, was given subcutaneously. The binding of ^{109}Cd in proteins of subcellular fractions of renal cells was studied 1 hr and 4 hr after the challenge dose.

Measured concentrations of Cd in the renal cortex and the distribution among subcellular fractions in animals from various groups are shown in Table 1. Renal-cortical Cd concentrations are similar to those previously reported at 24 hr after CdMT injection (10) and no obvious differences among groups related to pretreatment can be seen in total cortical Cd concentrations. There is a tendency towards a displacement of Cd from other fractions into the cytosol in the pretreated animals, but the proportions recovered in "membrane" and "microsome" fractions are not much different between pretreated and nonpretreated animals.

The results of gel filtration of subcellular fractions are shown in Figure 2 and Table 2. Whereas the distribution of ^{109}Cd was similar at 1 hr after the injection in nonpretreated and pretreated animals (groups A and C) there was a clear difference at 4 hr (groups B and D). In the nonpretreated animals (group B), most of the radioactivity appeared in high molecular weight (HMW) fractions, while, in the pretreated animals (group D), a major proportion of ^{109}Cd appeared in two low molecular weight peaks, possibly corresponding to metallothionein in monomeric and dimeric form. This is shown also in Table 2, where the fractional distribution of total ^{109}Cd in the various subcellular fractions is given. When considering the proportion of total membrane or microsome ^{109}Cd that is found in the HMW-fraction, there is no important difference between pretreated and nonpretreated animals at 1 hr, but a marked difference with 88% ("membrane") and 87% ("microsome") in HMW

Table 1. Total concentration of ^{109}Cd in renal cortex, and distribution of ^{109}Cd among subcellular fractions in various groups.[a]

Group	A	B	C	D
Pretreatment +/–	–	–	+	+
Survival time, hr	1 hr	4 hr	1 hr	4 hr
Total concentration of ^{109}Cd, μg/g wet wt	9.86	23.4	10.4	21.0
Nuclear, %	17	25	9	15
Mitochondria, %	12	7	11	5
Lysosome, %	9	8	10	9
Membrane, %	3	4	2	3
Microsome, %	3	4	3	3
Cytosol, %	56	52	65	65

[a] Percent of total amount in homogenate.
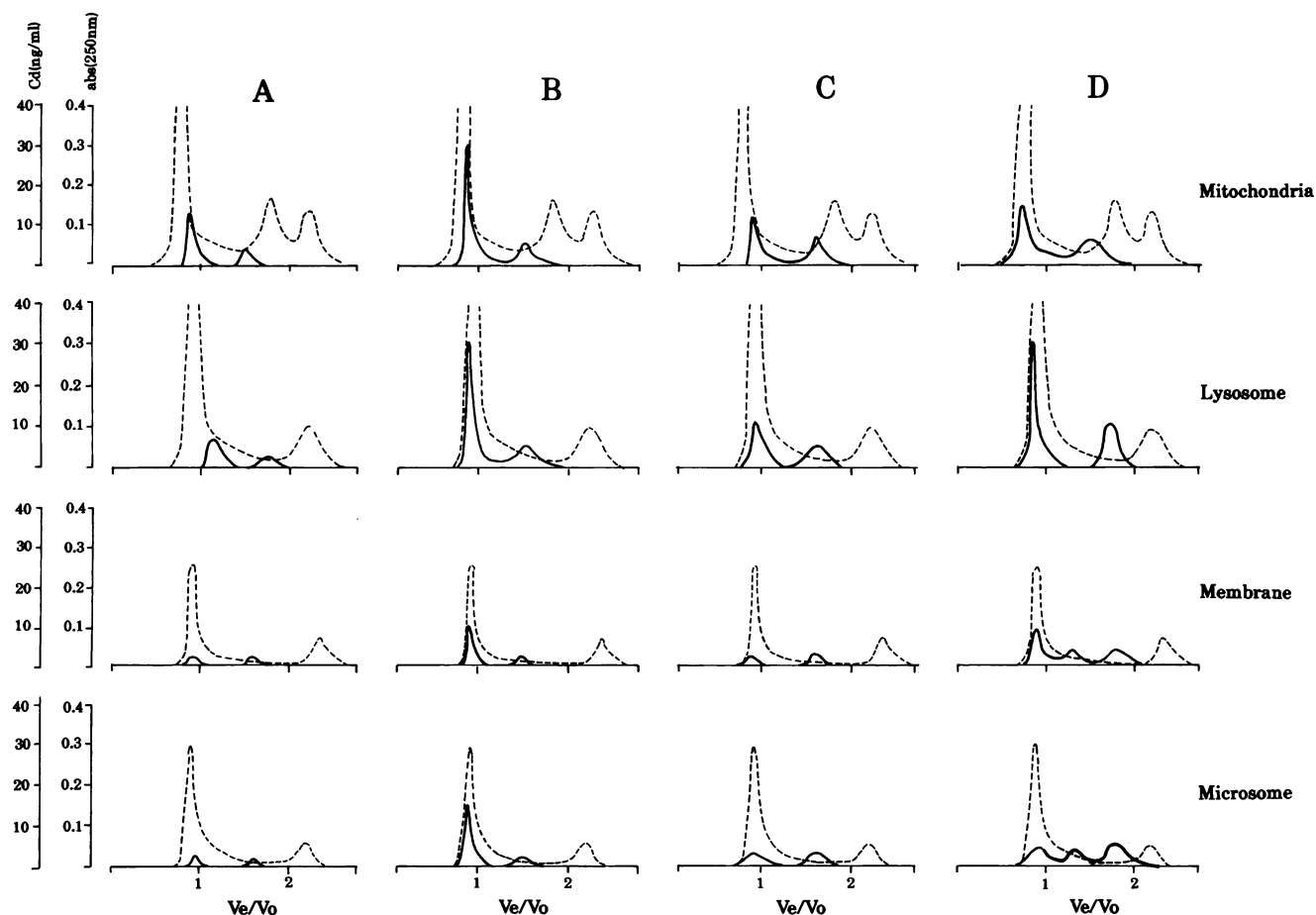
Figure 2. Gel chromatography on G-75 Sephadex of subcellular fractions from kidney cortex of rats in nonpretreated groups (A and B) and $CdCl_2$-pretreated groups (C and D, 0.5, 1, 1, 2, and 2 mg Cd/kg as $CdCl_2$ subcutaneously) 1hr (groups A and C) and 4 hr (groups B and D) after a challenge dose of CdMT (0.4 mg Cd/kg as CdMT, sc). Column dimensions were 26 x 364 mm. Elution was carried out with 0.01 M Tris-HCl 0.05 M NaCl buffer, pH 7.5, containing 0.1% DOC and performed at 4°C. A portion of each fraction (3.5 ml) was assayed for absorbance at 250 nm (- - - -) and radioactivity of [109]Cd (——). Ve/Vo of the peaks No. 1, 2, and 3 are 0. 80–1. 20, 1. 20–1.40, and 1. 60–2.10, respectively.

fraction at 4 hr in nonpretreated animals, compared to only 49% ("membrane") and 28% ("microsome") in the pretreated animals.

Discussion

Employing the [109]CdMT nephrotoxicity model, the present studies demonstrated a clear difference in [109]Cd binding to membrane proteins depending on whether or not metallothionein synthesis had been induced by pretreatment with nonradioactive Cd. This difference in binding occurred at 4 hr after injection of the toxic agent ([109]CdMT). Experimental conditions in the present study were identical with those used previously (*10*) to demonstrate that induction of metallothionein synthesis by repeated injections of $CdCl_2$ gives protection against the nephrotoxicity normally encountered after an sc injection of CdMT at the dose 0.,4 mg Cd/kg. It has been assumed in these previous studies that

the increased intracellular concentrations of metallothionein, induced by the pretreatment, would constitute the biochemical background for protection by sequestering Cd^{2+} ions from interaction with sensitive intracellular target sites. However, these targets have not been identified and the protective mechanism involving metallothionein hitherto has remained unproven.

The observations in the present study provide new insights into these mechanisms, both the one behind expression of toxicity and the protective mechanism. The findings are in agreement with a hypothesis implying that sensitive targets of importance for development of Cd nephrotoxicity are present in the HMW part of the membrane and microsome fractions of renal

Table 2. Percentage of [109]Cd recovered from G75 column bound in each protein peak in nonpretreated and pretreated groups after challenge dose of CdMT[a]

Group	A		B		C		D		
Peak number	1	3	1	3	1	3	1	2	3
Mitochondria	0.74	0.26	0.85	0.15	0.59	0.41	0.55		0.45
Lysosome	0.67	0.33	0.82	0.18	0.52	0.48	0.67		0.33
Membrane	0.50	0.50	0.88	0.12	0.53	0.47	0.49	0.21	0.30
Microsome	0.58	0.42	0.87	0.13	0.56	0.44	0.28	0.28	0.44

[a] Nonpretreated, groups A and B; treated, groups C and D. Testing 1 hr after challenge dose, groups A and C; testing 4 hr after challenge dose, groups B and D. Challenge dose of CdMT, 0.4 mg Cd/kg, administered subcutaneously. The ratio values of Ve/Vo for peaks 1,2, and 3 are 0.80–1.20, 1.20–1.40, and 1.60–2.10, respectively.

cortical cells and that the displacement of Cd from these sites by competitive binding to metallothionein (and possibly other low molecular weight proteins) is responsible for the protection of the pretreated animals from development of toxicity.

The timing of these observations, i.e., that the change in Cd binding to membrane proteins is seen 4 hr after injection of the toxic agent (CdMT) is in accordance with previous observations (11,12) from our laboratory demonstrating that increased calciuria starts 4 to 8 hr after CdMT injection. This early manifestation of Cd nephrotoxicity occurs several hours before tubular proteinuria appears. The fact that the changed membrane binding of Cd occurs simultaneously with the change in the earliest indicator of tubular toxicity

(calciuria), gives further support to the idea that there may be a causal link between Cd binding to targets in the membrane fraction and development of toxicity.

It has been demonstrated that both uptake and binding of Ca is impaired in luminal as well as basolateral membranes of renal tubule of rats in the same experimental model (11) in which calciuria is also seen. The Ca ion plays a central role in the regulation of many vital cell functions (16). It seems clear that a disruption of intracellular Ca^{2+} homeostasis can potentially lead to uncontrolled changes in such functions, which may result in toxicity. Cytosolic Ca^{2+} is normally maintained by the connected operation of transport systems located in the plasma membrane, endoplasmic reticulum, and mitochondria

(17). Cadmium is transported across cell membranes by Ca^{2+} transports and Ca^{2+} channels. Furthermore, the presence of Cd on these Ca^{2+} channel proteins, i.e., the subcellular fraction proteins, affect Ca^{2+} movement at these sites (18). The data presented support a hypothesis implying the following events: w°hen renal tubular cells contain sufficient amounts of metallothionein to bind a major proportion of Cd released from the catabolized CdMT taken up from tubular fluid, no toxicity occurs. However, if insufficient metallothionein protection is present, Cd interferes with luminal and basolateral plasma membranes and/or microsome function, with subsequent perturbations in Ca transport, leading to increased urinary Ca and increased intracellular calcium.

REFERENCES

1. Friberg L, Kjellström, T, Nordberg GF. Cadmium. In: Handbook on the Toxicology of Metals, 2nd ed. (Friberg L, Nordberg GF, Vouk V, eds). Amsterdam:Elsevier,1986; 130–184.
2. Nordberg M. Studies on metallothionein and cadmium. Environ Res 381–404 (1978).
3. Nordberg M, Nordberg GF. On the role of metallothionein in cadmium induced renal toxicity. Experientia (Suppl) 52:669–675 (1987).
4. Fowler BA, Nordberg GF. The renal toxicity of cadmium metallothionein: morphometric and X-ray microanalytical studies. Toxicol Appl Pharmacol 46:609–623 (1978).
5. Squibb KS, Ridlington JW, Carmichael NG, Fowler BA. Early cellular effects of circulating cadmium-thionein on kidney proximal tubules. Environ Health Perspect 28:287–296 (1979).
6. Squibb KS, Pritchard JB, Fowler BA. Renal metabolism and toxicity of metallothionein. In: Biological Roles of Metallothionein (Foulkes EC, ed). New York:Elsevier, 1982; 181–192.
7. Nordberg GF, Goyer RA, Nordberg M. Comparative toxicity of cadmium-metallothionein and cadmium chloride on mouse kidney. Arch Pathol 99:192–197 (1975).
8. Cherian MG, Goyer RA, Delaquerriere-Richardson L. Cadmium-metallothionein-induced nephrotoxicity. Toxicol Appl Pharmacol 38:399–408 (1976).
9. Webb M, Etienne AT. Studies on the toxicity and metabolism of cadmium-thionein. Biochem Pharmacol 26:25–30 (1977).

10. Jin T, Nordberg GF, Nordberg M. Resistance to acute nephrotoxicity induced by cadmium-metallothionein, dependence on pretreatment with cadmium chloride. Pharmacol Toxicol 61:89–93 (1987).
11. Jin T, Leffler P, Nordberg GF. Cadmium-metallothionein nephrotoxicity in the rat: transient calcuria and proteinuria. Toxicology 45:307–317 (1987).
12. Jin T, Nordberg M, Nordberg GF. Modulation of calciuria by cadmium pretreatment in rats with cadmium-metallothionein induced nephrotoxicity. Toxicology 75:29–37 (1992).
13. Maunsbach AB. Isolation of kidney lysosomes. Methods Enzymol, part A 31:330–333 (1974).
14. Nordberg GF, Nishiyama K. Whole body and hair retention of cadmium in mice. Arch Environ Health 24:209–214 (1972).
15. Nordberg M, Nordberg GF, Piscator M. Isolation and characterization of a hepatic metallothionein from mice. Environ Physiol Biochem 5:396–403 (1975).
16. Orrenius S, McConkey DJ, Jones DP, Nicotera P. Ca^{2+}-activated mechanisms in toxicity and programmed cell death. ISI Atlas of Science. Pharmacology 319–324 (1988).
17. Carafoli E, Crompton M. The regulation of intracellular calcium by mitochondria. Ann NY Acad Sci 307:269–284 (1978).
18. Hinkle PM, Kinsella PA, Osterhoudt KC. Cadmium uptake and toxicity via voltage-sensitive calcium channels. J Biol Chem 262:16333–16337 (1987).